Trachycladindoles A–G: cytotoxic heterocycles from an Australian marine sponge, *Trachycladus laevispirulifer*†

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Received 28th February 2008, Accepted 8th May 2008
First published as an Advance Article on the web 29th May 2008
DOI: 10.1039/b803455a

A southern Australian marine sponge, *Trachycladus laevispirulifer*, yielded the cytotoxic agents trachycladindoles A–G (**1–7**) as a selection of novel indole-2-carboxylic acids bearing a 2-amino-4,5-dihydroimidazole moiety. The trachycladindoles displayed promising selective cytotoxicity against a panel of human cancer cell lines and their structures were assigned on the basis of detailed spectroscopic analysis. Preliminary structure activity relationship (SAR) investigations by co-metabolite defined structural features key to the trachycladindole pharmacophore, highlighting an unusual bioactive molecular motif deserving of future investigation.

Introduction

During an investigation into anticancer agents from marine organisms, we examined a collection of invertebrates and algae acquired over a period of more than two decades from various southern Australian and Antarctic locations, ranging from intertidal to deep water, and utilizing collection methods from hand (SCUBA) to commercial and scientific trawling. Solvent extracts prepared from these samples were screened for their growth inhibitory and cytotoxic activity against a panel of human cancer cell lines, leading to the identification of a number of noteworthy bioactive extracts. Detailed chemical analysis of one of these extracts led to the identification of a novel family of cytotoxic indole-2-carboxylic acid heterocycles, trachycladindoles A–G (**1–7**). This report describes the isolation, characterization and structure elucidation of the trachycladindoles, together with preliminary structure activity relationship investigations.

Results and discussion

Several thousand marine specimens from multiple field collection sites were transferred frozen to the laboratory where they were catalogued, diced and steeped in aqueous EtOH at −30 °C for prolonged storage. To facilitate handling and biological screening, a portion (7 mL) of each extract was decanted, concentrated *in vacuo*, weighed and partitioned *in situ* between n-BuOH (2 mL) and H_2O (2 mL). Aliquots (1 mL) of both the n-BuOH and H_2O partitions were transferred to deep 96 well plates and stored at −30 °C ahead of being sub-sampled to support biological screening.

Screening aliquots (5–10 μL) of n-BuOH and H_2O partitions (2 × 2624 samples) were assayed against a selection of cancer cell lines to reveal 110 marine specimens with promising cytotoxicity profiles. Of these target specimens, 81 localized the cytotoxic activity exclusively in the n-BuOH partition, 23 exclusively in the H_2O partition, and 6 in both n-BuOH and H_2O partitions. This pool of targeted marine specimens formed the basis of our chemical investigations aimed at discovering new anticancer agents. In this report, we describe the discovery of novel cytotoxic metabolites, trachycladindoles A–G (**1–7**) produced by a specimen of *Trachycladus laevispirulifer* collected in 2001 during commercial trawling operations (Orange Roughy bycatch) in the Great Australian Bight. A portion of the aqueous EtOH extract obtained from a southern Australian specimen of *T. laevispirulifer* displaying cytotoxicity against human cancer cell lines was concentrated *in vacuo* and the residue (1.8 g) partitioned into n-BuOH (331 mg) and H_2O (1298 mg) solubles. The cytotoxic n-BuOH solubles were sequentially triturated with hexane, CH_2Cl_2 and MeOH, followed by reverse phase HPLC, to yield novel heterocyclic indole-2-carboxylic acids, trachycladindoles A–G (**1–7**).

High resolution ESI(+)MS analysis of trachycladindole A (**1**) revealed a pseudo molecular ion (M + H) consistent with a molecular formula ($C_{13}H_{13}BrN_4O_2$, Δmmu 1.4) incorporating nine double bond equivalents (DBE). Analysis of the ^{13}C NMR (CD_3OD) data for **1** (see Table 1) revealed eight sp^2 carbons (δ_C 112.5 to 135.1) attributed to four carbon–carbon double bonds, and a further two deshielded resonances (δ_C 168.6 and 160.4) consistent with sp^2 carbonyl/iminyl carbons. These observations accounted for six DBE and required that **1** be tricyclic. Further analysis of the ^1H and COSY NMR (CD_3OD) data revealed resonances and correlations consistent with three isolated spin systems; (a) a 1,2,4-trisubstituted aromatic ring (δ_H 7.30, dd, J = 8.7 and 1.8 Hz, H-6; 7.39, dd, J = 8.7 and 0.5 Hz, H-7; and 7.60, brs, H-4); (b) an *N*-methyl (δ_H 2.74, s, N12-Me); and (c) a deshielded methine–methylene pair (δ_H 6.38, brs, H-8; 3.69, brm, H-9b; and 4.06, dd, J = 10.2 and 10.2 Hz, H-9a). Deshielded ^{13}C NMR (CD_3OD) chemical shifts for C-8 (δ_C 58.9) and C-9 (δ_C 48.8), together with gHMBC correlations from H-9b and N12-Me to a common C-11 guanidino carbon (δ_C 160.4) and C-8, supported assignment of the 2-amino-4,5-dihydroimidazole heterocycle as shown. Such heterocycles are rare among published natural products with a noteworthy marine occurrence being discodermindole

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† Electronic supplementary information (ESI) available: Tabulated 1D and 2D NMR data, and ^1H NMR spectra for trachycladindoles A–G (**1–7**). See DOI: 10.1039/b803455a

Table 1 NMR (600 MHz, CD_3OD) data for trachycladindole A (**1**)

1				
#	δ_H/m	δ_C [b]	COSY	gHMBC (^1H-^{13}C)
*N*1-H	[a]			
2		[a]		
C2-CO_2H	[a]	168.6		
3		112.5		
3a		128.1		
4	7.60 (brs)	122.8	H-6	
5		114.3		
6	7.30 (dd)[c]	127.4	H-7, H-4	C-7a, C-5, C-4
7	7.39 (dd)[d]	115.5	H-6	C-6, C-5, C-4, C-3a
7a		135.1		
8	6.38 (brs)	58.9		
9a	4.06 (dd)[e]	48.8	H-9b	C-11, C-8, C-3
9b	3.69 (brm)		H-9a	
*N*10-H	[a]			
11		160.4		
*N*12-Me	2.74 (s)	29.7		C-11, C-8

[a] Not observed. [b] ^{13}C NMR assignments supported by DEPT and HSQC experiments. [c] 8.7, 1.8. [d] 8.7, 0.5. [e] 10.2, 10.2.

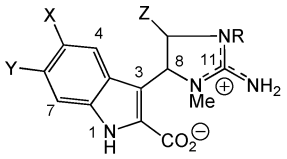
(**8**), reported in 1991 by Sun *et al.*[1] from a deep-water collection of the sponge *Discodermia polydiscus.* A second example of this structure class, 6-hydroxydiscodermindole (**9**), was reported by Gunasekera *et al.*[2] during a 2004 re-investigation of this same *D. polydiscus* specimen. A significant difference in the NMR (d_6-DMSO) data for the 2-amino-4,5-dihydroimidazole heterocycle in trachycladindole A (**1**) compared to discodermindole (**8**) was the pronounced deshielding of H-8 ($\Delta\delta_H$ −1.21) and C-8 ($\Delta\delta_C$ −5.3). This deshielding was attributed to the ability of **1** to form an internal salt with restricted conformational freedom (see below). Further spectroscopic comparison to **8** permitted incorporation of the 1,2,4-trisubstituted aromatic structure fragment in **1** into the indole ring system as shown. Placement of the 2-amino-4,5-dihydroimidazole substituent at C-3 (as indicated) was supported by gHMBC (CD_3OD) correlations (see Table 1) from H-9a to an sp^2 carbon resonance attributed to C-3 (δ_C 112.6), and further confirmed by a suite of diagnostic NOESY (CD_3OD) correlations between H-9a, H-9b and N12-Me, to H-4 (δ_H 7.60). These NOESY correlations together with further NMR comparisons to **8**, also confirmed placement of a C-5 bromo substituent as indicated. The structure arguments as presented above for **1** account for all but the identity of the C-2 substituent and the elements of CO_2H. As a result, the complete planar structure for trachycladindole A (**1**) was proposed as shown (in an internal salt form—see below).

High resolution ESI(+)MS analysis of trachycladindole B (**2**) revealed a pseudo molecular ion (M + Na) consistent with a molecular formula ($C_{14}H_{15}BrN_4O_2$, Δmmu −0.3) for a methyl homologue of trachycladindole A (**1**). Comparison of the NMR (CD_3OD) data between **1** and **2** (see Tables 1, 2) revealed the only significant difference to be an additional *N*-methyl resonance (δ_H 3.11 and δ_C 32.5) in **2** compared to **1**. Further analysis of the gHMBC (CD_3OD) data revealed key correlations between the N12-Me and C-8, and the N10-Me and flanking carbons C-9 and C-11. These observations together with diagnostic NOESY (CD_3OD) correlations between H-9b, N10-Me and N12-Me, to H-4 (δ_H 7.56), confirmed the planar structure for trachycladindole B (**2**) as shown (in an internal salt form—see below).

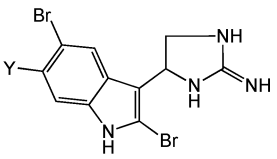
Table 2 ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) data from trachycladindoles B–G (**2–7**)

	2		3		4		5		6		7	
#	δ_H/m, *J*/Hz	δ_C [b]	δ_H/m *J*/Hz	δ_C [b]	δ_H/m *J*/Hz	δ_C [b]	δ_H/m *J*/Hz	δ_C [b]	δ_H/m *J*/Hz	δ_C [b]	δ_H/m *J*/Hz	δ_C [b]
*N*1-H	[a]		[a]		[a]		[a]		[a]		[a]	
2		[a]		135.2[c]		[a]		[a]		137.5[c]		[a]
C2-CO_2H	[a]	168.5	[a]	169.2	[a]	169.0	[a]	168.2[c]	[a]	168.9[c]	[a]	169.2[c]
3		111.9		112.9		112.2		[a]		110.9[c]		112.6
3a		128.6		121.2		121.3		122.1[c]		120.9[c]		126.5[c]
4	7.56 (brs)	122.9	7.55 (s)	124.2	7.49 (brs)	124.1	[a]	[a]	7.25 (brs)	123.3[c]	7.46 (d, 8.4)[d]	120.4[c]
5		114.2		106.0		106.3[c]		114.4[c]		106.9	7.05 (bdd, 7.5, 7.6)	121.2[c]
6	7.30 (dd, 8.7, 1,8)	127.4		152.3		152.5[c]	7.29 (dd, 8.7, 1.7)	127.4		153.4	7.21 (bdd, 7.5, 7.6)	124.7[c]
7	7.39 (d, 8.7)	115.5	6.99 (s)	99.2	6.98 (s)	99.4	7.39 (d, 8.7)	115.5	6.95 (brs)	99.1	7.46 (d, 8.4)[d]	113.8[c]
7a		135.2		137.5		135.4[c]		135.0[c]		137.4		137.1[c]
8	6.25 (brs)	57.1	6.35 (brs)	58.9	6.24 (brs)	57.2	6.06 (brs)	64.6	6.09 (brs)	65.4	6.42 (brs)	59.1
9a	4.06 (dd, 9.9, 9.9)	55.9	4.04 (dd, 10.1, 10.1)	48.2	4.03 (dd, 10.0, 10.0)	55.9	5.29 (brs)	86.4	5.28 (brs)	89.9[c]	4.06 (dd, 10.0, 10.0)	48.4
9b	3.71 (brm)		3.68 (brm)		3.67 (brm)						3.75 (dd, 8.8, 8.8)	
*N*10-Me	3.11	32.5			3.10 (s)	32.5	3.11 (s)	29.3	3.13 (s)	28.9		
11		159.8		160.3		159.7		158.0		158.1		160.3
*N*12-Me	2.75 (s)	30.6	2.74	29.7	2.74 (s)	30.5	2.79 (s)	30.2	2.80 (s)	29.8	2.73 (s)	29.6

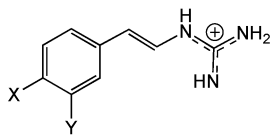
[a] Not observed. [b] ^{13}C NMR assignments supported by HSQC. [c] Assignment tentative. [d] Overlapping resonances.



	X	Y	Z	R
(1)	Br	H	H	H
(2)	Br	H	H	Me
(3)	Br	OH	H	H
(4)	Br	OH	H	Me
(5)	Br	H	OH	Me
(6)	Br	OH	OH	Me
(7)	H	H	H	H



	Y
(8)	H
(9)	OH



	X	Y
(10)	OH	OH
(11)	OH	H

High resolution ESI(+)MS analysis of trachycladindole C (**3**) revealed a pseudo molecular ion (M + H) consistent with a molecular formula ($C_{13}H_{13}BrN_4O_3$, Δmmu +0.9) for an oxy analogue of trachycladindole A (**1**). Comparison of the ^{1}H NMR (CD_3OD) data between **1** and **3** (see Tables 1, 2) revealed the only significant difference to be a simplification of the aromatic spin system to one consisting of two isolated protons (δ_H 6.99 and 7.55). NMR (CD_3OD) comparisons between **3** and the known sponge metabolite 6-hydroxydiscodermindole (**9**) permitted placement of a 6-hydroxy substituent and allowed assignment of the planar structure for trachycladindole C (**3**) as shown (in an internal salt form—see below).

High resolution ESI(+)MS analysis of trachycladindole D (**4**) revealed a pseudo molecular ion (M + H) consistent with a molecular formula ($C_{14}H_{15}BrN_4O_3$, Δmmu +0.9) for a methyl homologue of trachycladindole C (**3**). Excellent NMR (CD_3OD) comparisons between **4** and co-metabolites **1–3** (see Tables 1, 2) permitted assignment of the planar structure for trachycladindole D (**4**) as shown (in an internal salt form—see below).

An unexpected spectroscopic feature in common between trachycladindoles A–D (**1–4**) was pronounced broadening of the ^{1}H NMR resonances for H-4, H-8 and H-9b, and dramatic deshielding of H-8. This effect was evident in both CD_3OD and d_6-DMSO but, significantly, was not reported for the known discodermindoles **8** and **9**. Taking note of potential spatial relationships between these unusually broadened and deshielded protons, we hypothesize that this effect is a consequence of "internal salt" formation. Intramolecular ionic association between guanidinium and carboxylate moieties could constrain orientation of the 2-amino-4,5-dihydroimidazole heterocycle relative to the plane of the indole ring, positioning H-8 within the deshielding zone of the aromatic system and placing H-4, H-8 and H-9b (and on occasion H-9a, N10-Me and N12-Me) in a proximity sufficient to explain observed NOESY correlations (see Fig. 1). Such conformational constraint notwithstanding, limited rotational flexibility about the 3–8 bond could lead to H-4, H-8 and H-9b experiencing a

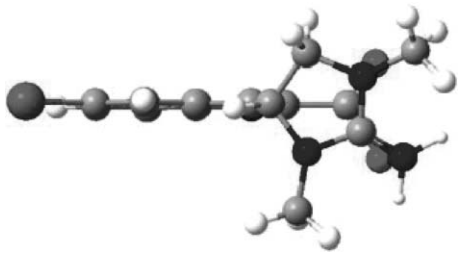


Fig. 1 3D (Chem 3D) representation of trachycladindole B (**2**) in the proposed internal salt form.

spectrum of steric interactions and magnetic environments that could manifest as broadened ^{1}H NMR resonances.

High resolution ESI(+)MS analysis of trachycladindole E (**5**) revealed a pseudo molecular ion (M + H) consistent with a molecular formula ($C_{14}H_{15}BrN_4O_3$, Δmmu +0.8) isomeric with trachycladindole D (**4**). However, unlike **4**, the NMR (CD_3OD) data for **5** (see Table 2) lacked evidence of a phenolic hydroxy substituent—instead revealing an indole substitution in common with trachycladindoles A (**1**) and B (**2**). Further analysis of the ^{1}H NMR data for **5** revealed resonances consistent with conversion of the 2-amino-4,5-dihydroimidazole methylene in **4** (H-9a and H-9b) to a hydroxymethine in **5** (H-9, δ_H 5.29; δ_C 86.4), along with resonances for two guanidino *N*-methyls (δ_H 2.79 and 3.11) with gHMBC correlations to a common guanidino sp^2 iminyl carbon (δ_C 158.0) as well as C-8 (δ_C 64.6) and C-9 (δ_C 86.4) respectively. In common with **1–4**, the ^{1}H NMR data for **5** exhibited pronounced broadening of resonances for H-4, H-8 and H-9 to the point where the resonance for H-8 was barely detectable while that for H-4 was rendered undetectable. [Note—the existence of H-4 could be inferred from an m-coupling with H-6 ($J_{4,6}$ = 1.7 Hz), while the existence of H-8 could be inferred from H-8 HSQC and N12-Me gHMBC correlations to C-8 (δ_C 65.1).] The challenges in interpreting broadened NMR resonances notwithstanding, the spectroscopic data for **5** was consistent with a C-9 hydroxy analogue of trachycladindole B (**2**). Furthermore, in light of the hypothesis presented above for ^{1}H NMR broadening, and given that H-9 in **5** is significantly broadened, we tentatively propose that H-9 in **5** adopts the same relative stereochemistry to H-8 as does H-9b in **1–4**. Thus the structure for trachycladindole E (**5**) was proposed as shown.

High resolution ESI(+)MS analysis of trachycladindole F (**6**) revealed a pseudo molecular ion (M + H) consistent with a molecular formula ($C_{14}H_{15}BrN_4O_4$, Δmmu 0.0) for an oxy analogue of trachycladindole E (**5**). Again, as with all other trachycladindoles, ^{1}H NMR resonances for H-4, H-8 and H-9 were significantly broadened, to the point where the former two resonances were barely detectable. [Note—while the ^{1}H NMR resonances for H-4 and H-8 were extremely broad, they were detectable and could be integrated—they did not, however, provide 2D NMR correlations in either COSY, NOESY, HSQC or gHMBC experiments.] The challenges in interpreting broadened ^{1}H NMR resonances notwithstanding, analysis of the available spectroscopic data for **6** (see Table 2) supported the presence of a 5-bromo-6-hydroxyindole-2-carboxylic acid moiety in common with **3** and **4**, and an *N*,*N*-dimethyl 2-amino-4-hydroxy-4,5-dihydroimidazole moiety in common with **5**. Significant broadening of the ^{1}H NMR resonances for H-9 was consistent with and permitted tentative

assignment of a relative stereochemistry in common with **5**. Thus the structure for trachycladindole F (**6**) was proposed as shown.

High resolution ESI(+)MS analysis of trachycladindole G (**7**) revealed a pseudo molecular ion (M + H) consistent with a molecular formula ($C_{13}H_{14}N_4O_2$, Δmmu +0.7) for a debromo analogue of trachycladindole A (**1**). Particularly diagnostic in the ^{1}H NMR (CD_3OD) data for **7** were resonances attributed to H-4/H-7 (δ_H 7.46, d) and H-5/H-6 (δ_H 7.21, dd, 7.5 and 7.6 Hz; 7.05, dd, 7.5 and 7.6 Hz) of an indole, together with a broadened and deshielded H-8 resonance (δ_H 6.42, br) common to **1–6**. Furthermore, and common with **1**, trachycladindole G (**7**) displayed an *N*-methyl resonance (δ_H 2.73, s) with NOESY and HMBC correlations to H-8, C-8 and C-11, supporting an N12 methyl regiochemistry. As with other trachycladindoles, the ^{1}H NMR (CD_3OD) data for **7** revealed a highly deshielded H-8 resonance, along with significant broadening of H-8 and H-9b. Overlapping ^{1}H NMR (CD_3OD) resonances for H-4 and H-7 precluded observation of the typical trachycladindole broadened H-4 resonance, however, reacquisition of the ^{1}H NMR data in d_6-DMSO resolved these two overlapping resonances and revealed H-4 to be, as expected, highly broadened (δ_H 7.35, brs). These observations, together with analysis of the complete 2D NMR data set allowed assignment of the planar structure for trachycladindole G (**7**) as shown.

As presented above, the trachycladindoles A–G (**1–7**) possess new molecular structures incorporating a rare heterocyclic moiety, and are proposed to exist preferentially as internal salts with pronounced deshielding and broadening of selected ^{1}H NMR resonances. Efforts to disconnect the internal salt and reduce NMR broadening by methylation of the carboxylic acid proved unsuccessful. Repeated attempts at methylation of trachycladindole C (**3**) with MeI–DMF yielded the expected 6-OMe derivative, but did not yield the desired methyl ester. Likewise, repeated efforts at methylation of trachycladindole D (**4**) with CH_2N_2 did not result in a methyl ester. These results suggest that the internal salt is particularly stable. With regards to stability, it is interesting to note that on prolonged storage in DMSO (>12 months) trachycladindole E (**5**) undergoes conversion to a 1 : 1 mixture with trachycladindole A (**1**). The mechanism for a formal loss of HCHO remains unclear at this time. Trachycladindole F (**6**) also proved to be unstable to long term storage in DMSO, however, the identity of the decomposition product(s) remains unknown.

Biosynthetically, the trachycladindoles (**1–7**) are closely related to the discodermindoles (**8–9**), with both likely derived from a tryptophan precursor. A putative biosynthesis (see Fig. 2) proposes parallel pathways to discodermindoles and trachycladindoles. The discodermindole pathway could progress *via* C-2 bromination of tryptophan, elimination of HCO_2H and conversion of the amino to a guanidino functionality, to return a guanidinoenamine which undergoes an intramolecular enzyme mediated (stereochemically controlled) E2 addition to yield the 2-amino-4,5-dihydroimidazole heterocycle as shown. Subsequent action of bromoperoxidase/oxidase enzymes would complete the transformation to discodermindoles (**8–9**). In the trachycladindole pathway, tryptophan undergoes intramolecular CO_2H transfer to C-2 *via* a cyclic intermediate (see Fig. 2) and conversion of the amino to a guanidino functionality to yield a guanidinoenamine which then undergoes an intramolecular enzyme mediated (stereochemically controlled) E2 addition, leading to the 2-amino-4,5-dihydroimidazole heterocycle as shown. Subsequent action of methyl transfer and bromoperoxidase/oxidase enzymes leads to trachycladindoles A–D (**1–4**) and G (**7**). In a subtle variation to this pathway, enzyme mediated (stereochemically controlled) epoxidation of the guanidinoenamine (see Fig. 2) followed by intramolecular S_N2 addition of guanidine to the epoxide could, subsequent to the action of bromoperoxidase/oxidase and methyl transfer enzymes, yield trachycladindoles E–F (**5–6**). In this biosynthetic proposal, the timing of C-2 bromination offers a mechanism for discriminating between discodermindole and trachycladindole pathways, although, alternative discrimination strategies are feasible.

The rationale behind proposing a plausible biosynthetic pathway is to illustrate that a limited repertoire of biosynthetic transformations could be sufficient to assemble the full suite

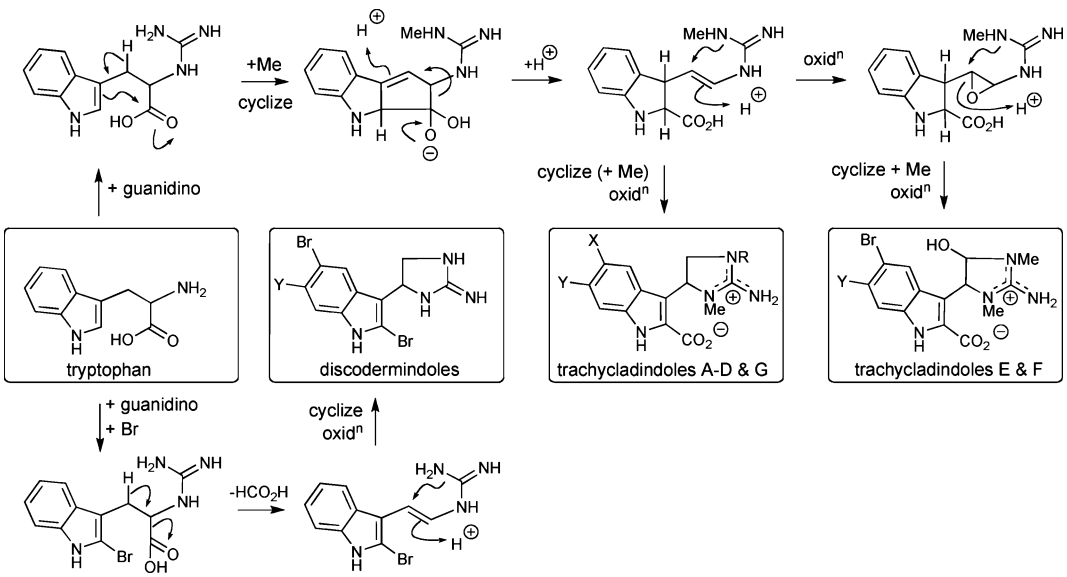


Fig. 2 Putative biosynthetic pathway.

of discodermindoles and trachycladindoles from a commonly available amino acid precursor. While naturally occurring tryptophan derived guanidinoenamines similar to those in the proposed biosynthesis have yet to be discovered, two closely related tyrosine derived guanidinoenamines have been reported from marine sources. Tubastrine (**10**) was first reported in 1987 by Higa *et al.*[3] as the antiviral agent from an Okinawan coral (*Tubastrea aurea*), and reappeared in a 2005 study by Köck *et al.*[4] into the EGFR inhibitor in a Scottish ascidian *Dendrodoa grossularia.* The guanidinoenamine **11** was reported in 1994 by Capon *et al.*[5] as the antibacterial agent from a southern Australian marine sponge *Spongosorites* sp. To the best of our knowledge, known natural occurrences of the 2-amino-4,5-dihydroimidazole moiety common to trachycladindoles and discodermindoles are limited to 1977[6] and 1978[7] reports of the plant seed amino acids enduracidin and 2-[2-amino-2-imidazolin-4-yl] acetic acid, a 2002 report[8] of the microbial glycopeptide antibacterial mannopeptimycins, and a 2005 report[9] of the ascidian metabolite tiruchanduramine. The paucity of literature reports reinforces the observation that naturally occurring 2-amino-4,5-dihydroimidazoles are rare, and that the trachycladindoles possess novel molecular structures. Although all trachycladindoles were chiral, the absolute stereochemistry, and in the case of **5** and **6** the relative stereochemistry, remain unassigned. Lack of naturally available material and demands for bioassays, together with stability issues, requires that confirmation of structures and stereochemical assignments best be achieved by total synthesis.

Experimental

General experimental procedures

Chiroptical measurements ($[\alpha]_D$) were obtained on a Jasco P-1010 Intelligent Remote Module polarimeter in a 100 by 2 mm cell. Ultraviolet (UV) absorption spectra were obtained using a CARY3 UV–visible spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were performed on either a Bruker Avance 600 spectrometer, in the solvents indicated, and referenced to residual ^{1}H signals in the deuterated solvents. Electrospray ionization mass spectra (ESIMS) were acquired using an Agilent 1100 Series Separations Module equipped with an Agilent 1100 Series LC/MSD mass detector in both +ve and −ve ion modes. High resolution (HR) ESIMS measurements were obtained on a Finnigan MAT 900 XL Trap instrument with a Finnigan API III source. HPLC was performed using an Agilent 1100 Series Separations Module equipped with Agilent 1100 Series Diode Array and/or Multiple Wavelength Detectors, and Agilent 1100 Series Fraction Collector, controlled using ChemStation Rev.9.03A and Purify version A.1.2 software.

Collection

The sponge sample (UQ code: CMB-03397) was collected during commercial trawling operations (Orange Roughy bycatch) aboard the Lucky S. in the Great Australian Bight (latitude: 33° 12′ S, longitude 128° 15′ E) in 2001. The fresh sample was frozen for shipping (−4 °C) to the laboratory, where it was thawed, catalogued, diced and steeped in aqueous EtOH at −30 °C for prolonged storage.

Taxonomy

Specimen CMB-03397 was described as follows: colour in life orange. Growth form erect, arborescent, branches cylindrical, dichotomously branching with rounded club-shaped ends, 5–10 mm diam. Texture spongy, flexible but difficult to tear. Oscules discrete and scattered over branches. Surface opaque, optically smooth, wrinkled in places. Spicules include oxeas curved, telescoped and occasionally stylote (300–580 × 7–10 μm); microscleres are spinispirae spined, c-shaped or double spiral (5–8 μm). Ectosome a distinct cortex, 100 μm thick, of densely packed spinispirae. Choanosome has a condensed axial region of fibres cored by oxeas becoming plumose tracts coring fibres in the extra-axial region. Collagen abundant extra-axially and filled with spinispirae. Specimen CMB-03397 was identified as class: demospongiae, order: hadromerida, family: trachycladidae, genus and species: *Trachycladus laevispirulifer* (Carter, 1879b), and a voucher sample was deposited with Museum Victoria (Reg No MVF147424).

Cytotoxicity assay

The *in vitro* cytotoxicity screening methodology carried out on lung (A549), colorectal (HT29) and breast (MDA-MB-231) cancer cell lines has been reported.[10]

Extraction and isolation

A portion of the aqueous EtOH extract obtained from a southern Australian *Trachycladus* sp. was concentrated *in vacuo* and the crude extract (1.8 g) partitioned between n-BuOH (0.33 g) and H_2O (1.30 g). The n-BuOH solubles were sequentially triturated with hexane (79.1 mg), CH_2Cl_2 (29.7 mg) and MeOH (204 mg). A portion of the MeOH solubles (50 mg) were fractionated by HPLC (4.0 mL min^{-1} 33 min gradient elution 90% H_2O–MeOH to MeOH through a Zorbax 5 μm C_8 250 × 10 mm column, followed by 4.0 mL min^{-1} 33 min gradient elution 50% H_2O–CH_3CN to 12.5% H_2O–CH_3CN through a Zorbax 5 μm CN 250 × 10 mm column) to yield trachycladindoles A (**1**) (3.1 mg, 0.16%*), B (**2**) (1.4 mg, 0.07%*), C (**3**) (4.3 mg, 0.22%*), D (**4**) (1.4 mg, 0.7%*), E (**5**) (5.9 mg, 0.3%*) and F (**6**) (2.8 mg, 0.15%*). The CH_2Cl_2 solubles were triturated with CH_3CN (21 mg) and MeOH (8 mg), and a portion of the MeOH solubles (3 mg) was fractionated by HPLC (1.0 mL min^{-1} 25 min gradient elution a 90% H_2O–CH_3CN to CH_3CN through a Zorbax 5 μm C_8 150 × 4.6 mm column) to yield trachycladindole G (**7**) (1.2 mg, 0.22%*). (* percentage yields calculated as weight to weight estimates in 1.8 g of crude extract.)

Trachycladindole A (1)

White solid; $[\alpha]_D$ +5.9 (*c* 0.95, MeOH); UV (MeOH) λ_{max} nm (log e) 225 (3.39), 296 (2.92); ^{1}H and ^{13}C NMR (CD_3OD) data see Table 1; ^{1}H NMR (600 MHz, d_6-DMSO) 7.53 (1H, brs, H-4), 7.38 (1H, d, 8.6 Hz, H-7), 7.24 (1H, dd, 8.5, 1.5 Hz, H-6), 6.44 (1H, brs, H-8), 3.98 (1H, dd, 9.8, 9.8 Hz, H-9a), 3.57 (1H, brs, H-9b), 2.69 (3H, s, N12-Me); ^{13}C NMR (150 MHz, d_6-DMSO) 168.6 (s), 160.4 (s), 135.1 (s), 128.1 (s), 127.4 (d), 122.8 (d), 115.5 (d), 114.3 (s), 112.5 (s), 58.9 (d), 48.8 (t), 29.7 (q); ESI(+)MS *m/z* 337/339 $[M + H]^+$; ESI(−)MS *m/z* 335/337 $[M - H]^-$; HRESI(+)MS *m/z* 337.0309 $[M + H]^+$ ($C_{13}H_{14}BrN_4O_2$ requires 337.0300).

Table 3 Cell cytotoxicity data for trachycladindoles A–G (**1–7**)

#		Lung (A549)/μM	Colorectal (HT29)/μM	Breast (MDA-MB-231)/μM
(**1**)	GI_{50}	6.5	2.9	1.2
	TGI	9.2	7.4	1.6
	LC_{50}	20.8	[a]	[a]
(**2**)	GI_{50}	1.3	0.5	2.7
	TGI	2.0	1.8	5.1
	LC_{50}	5.4	[a]	[a]
(**3**)	GI_{50}	19.8	4.8	12.2
	TGI	[a]	18.1	[a]
	LC_{50}	[a]	[a]	[a]
(**4**)	GI_{50}	1.7	0.4	2.4
	TGI	1.9	0.9	5.7
	LC_{50}	[a]	[a]	[a]
(**5**)	GI_{50}	0.5	0.3	1.1
	TGI	0.7	0.8	2.1
	LC_{50}	2.1	[a]	9.0
(**6**)	GI_{50}	1.2	0.8	2.3
	TGI	1.7	1.8	3.6
	LC_{50}	[a]	[a]	[a]
(**7**)	GI_{50}	[a]	[a]	[a]
	TGI	[a]	[a]	[a]
	LC_{50}	[a]	[a]	[a]

[a] Not detected at 30 μM.

Trachycladindole B (2)

White solid; $[\alpha]_D$ +8.8 (*c* 0.94, MeOH); UV (MeOH) λ_{max} nm (log e) 296 (2.60); ^1H and ^{13}C NMR (CD_3OD) data see Tables 2 and 3; ESI(+)MS *m/z* 351/353 $[M + H]^+$; ESI(−)MS *m/z* 349/351 $[M - H]^-$; HRESI(+)MS *m/z* 373.0268 $[M + Na]^+$ ($C_{14}H_{15}BrN_4NaO_2$ requires 373.0276).

Trachycladindole C (3)

White solid; $[\alpha]_D$ +2.9 (*c* 1.31, MeOH); UV (MeOH) λ_{max} nm (log e) 225 (3.24), 245 (3.04), 313 (3.12), 320 (3.10); ^1H and ^{13}C NMR (CD_3OD) data see Tables 2 and 3; ESI(+)MS *m/z* 355/357 $[M + H]^+$; ESI(−)MS *m/z* 353/355$[M - H]^-$; HRESI(+)MS *m/z* 353.0253 $[M + H]^+$ ($C_{13}H_{14}BrN_4O_3$ requires 353.0249).

Trachycladindole D (4)

White solid; $[\alpha]_D$ +7.5 (*c* 1.02, MeOH); UV (MeOH) λ_{max} nm (log e) 225 (3.29), 245 (2.85), 313 (2.90), 320 (2.88); ^1H and ^{13}C NMR (CD_3OD) data see Tables 2 and 3; ESI(+)MS *m/z* 367/369 $[M + H]^+$; ESI(−)MS *m/z* 365/367 $[M - H]^-$; HRESI(+)MS *m/z* 367.0398 $[M + H]^+$ ($C_{14}H_{16}BrN_4O_3$ requires 367.0406).

Trachycladindole E (5)

White solid; $[\alpha]_D$ +11.6 (*c* 0.48, MeOH); UV (MeOH) λ_{max} nm (log e) 225 (3.35), 296 (2.90); ^1H and ^{13}C NMR (CD_3OD) data see Tables 2 and 3; ESI(+)MS *m/z* 367/369 $[M + H]^+$; ESI(−)MS *m/z* 365/367 $[M - H]^-$; HRESI(+)MS *m/z* 367.0408 $[M + H]^+$ ($C_{14}H_{16}BrN_4O_3$ requires 367.0406).

Trachycladindole F (6)

White solid; $[\alpha]_D$ −4.8 (*c* 0.94, MeOH); UV (MeOH) λ_{max} nm (log e) 225 (3.36), 245 (3.13), 313 (3.21), 320 (3.18); ^1H and ^{13}C NMR (CD_3OD) data see Tables 2 and 3; ESI(+)MS *m/z* 383/385 $[M + H]^+$; ESI(−)MS *m/z* 381/383 $[M - H]^-$; HRESI(+)MS *m/z* 383.0349 $[M + H]^+$ ($C_{14}H_{16}BrN_4O_4$ requires 383.0355).

Trachycladindole G (7)

White solid; $[\alpha]_D$ +140.3 (*c* 0.60, MeOH); UV (MeOH) λ_{max} nm (log e) 225 (3.03), 292 (2.88); ^1H and ^{13}C NMR (CD_3OD) data see Tables 2 and 3; ^1H NMR (600 MHz, d_6-DMSO) 7.37 (1H, d, 8.2 Hz, H-7), 7.34 (1H, brd, 5.9 Hz, H-4), 7.08 (1H, dd, 7.5, 7.5 Hz, H-6), 6.95 (1H, dd, 7.5, 7.5 Hz, H-5), 6.53 (1H, brs, H-8), 3.91 (1H, brm, H-9a), 3.57 (1H, brm, H-9b), 2.64 (3H, s, N12-Me); ESI(+)MS *m/z* 259 $[M + H]^+$; ESI(−)MS *m/z* 257 $[M - H]^-$; HRESI(+)MS *m/z* 259.1197 $[M + H]^+$ ($C_{13}H_{15}BrN_4O_2$ requires 259.1195).

Conclusion

Sponges of the genus *Trachycladus* do not feature prominently in the natural products literature, with reports being limited to two Australian collections. These include a 1995 account by Molinski *et al.*[11] on the trachycladines, cytotoxic nucleosides obtained from a *T. laevispirulifer* specimen collected by SCUBA (−10 m) at Exmouth Gulf in Western Australia, and a 2001 account by Capon *et al.*[12] on onnamide F, a nematocidal and antifungal polyketide obtained from a *T. laevispirulifer* specimen collected by SCUBA (−15 m) in Port Phillip Bay, Victoria. This current study represents only the third reported occurrence of natural products from *T. laevispirulifer*, extending the two earlier reports of nucleosides and polyketides to include the trachycladindoles, indolyl alkaloids incorporating rare and unusual structural features.

A comparison of the cytotoxicity of trachycladindoles A–G (**1–7**) against lung (A549), colorectal (HT29), and breast (MDA-MB-231) cancer cell lines (see Table 3) revealed GI_{50} and TGI with sub μM potency. Of note, trachycladindole G (**7**) lacked significant cytotoxicity at +30 μM, highlighting the structure activity relationship (SAR) importance of C-5 bromination and/or C-6 hydroxylation. Similarly, comparative cytotoxicity data for trachycladindoles A–F (**1–6**) revealed a pharmacophore bias in favor of N10 and N12 dimethylation (*i.e.* lung and colorectal potency trends have **2** better than **1**, and **4** better than **3**) and C-9 hydroxylation (*i.e.* lung, colorectal and breast potency trends have **5** better than **1**, and **6** better than **2**). These assessments of the trachycladindole pharmacophore, while only preliminary, do point to clear relationships between structure and potency, and encourage the view that further investigation of this unusual pharmacophore is warranted.

Acknowledgements

The authors would like to thank K. Smith (Marine and Freshwater Research Institute, Victorian Department of Primary Industries) for assistance in specimen collection, L. Goudie for sponge taxonomy, C. Cuevas and A. Francesch (PharmaMar) for *in vitro* cell cytotoxicity screening, and G. MacFarlane (School of Molecular and Microbial Science, The University of Queensland) for the acquisition of HRESIMS data. This research was funded in part by the Australian Research Council, with support from PharmaMar (Madrid, Spain).

Notes and references

1 H. H. Sun and S. Sakemi, *J. Org. Chem.*, 1991, **56**, 4307–4308.
2 J. Cohen, G. K. Paul, S. P. Gunasekera, R. E. Longley and S. A. Pomponi, *Pharm. Biol.*, 2004, **42**, 59–61.
3 R. Sakai and T. Higa, *Chem. Lett.*, 1987, **1**, 127–128.
4 J. S. Barenbrock and M. Köck, *J. Biotechnol.*, 2005, **117**, 225–232.
5 S. Urban, R. J. Capon and J. N. A. Hooper, *Aust. J. Chem.*, 1994, **47**, 2279–2282.
6 L. E. Fellows, R. C. Hider and E. A. Bell, *Phytochemistry*, 1977, **16**, 1957–1959.
7 L. E. Fellows, R. M. Polhill and E. A. Bell, *Biochem. Syst. Ecol.*, 1978, **6**, 213–215.
8 H. Y. He, R. T. Williamson, B. Shen, E. I. Graziani, H. Y. Yang, S. M. Sakya, P. J. Petersen and G. T. Carter, *J. Am. Chem. Soc.*, 2002, **124**, 9729–9736.
9 K. Ravinder, A. V. Reddy, P. Krishnaiah, P. Ramesh, S. Ramakrishna, H. Laatsch and Y. Venkateswarlu, *Tetrahedron Lett.*, 2005, **46**, 5475–5478.
10 J. Tulla-Puche, N. Bayo-Puxan, J. A. Moreno, A. M. Francesch, C. M. Alvarez and F. Albericio, *J. Am. Chem. Soc.*, 2007, **129**, 5322–5323.
11 P. A. Searle and T. F. Molinski, *J. Org. Chem.*, 1995, **60**, 4296–4298.
12 D. Vuong, R. J. Capon, E. Lacey, J. H. Gill, K. Heiland and T. Friedel, *J. Nat. Prod.*, 2001, **64**, 640–642.